

RESEARCH PAPER

Short-term treatment with the GABA_A receptor antagonist pentylenetetrazole produces a sustained pro-cognitive benefit in a mouse model of Down's syndrome

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BACKGROUND AND PURPOSE

Down's syndrome is a common genetic cause of intellectual disability, for which there are no drug therapies. Mechanistic studies in a model of Down's syndrome [Ts65Dn (TS) mice] demonstrated that impaired cognitive function was due to excessive neuronal inhibitory tone. These deficits were normalized by low doses of GABA_A receptor antagonists in adult animals. In this study, we explore the therapeutic potential of pentylenetetrazole, a GABA_A receptor antagonist with a history of safe use in humans.

EXPERIMENTAL APPROACH

Long-term memory was assessed by the novel object recognition test in different cohorts of TS mice after a delay following a short-term chronic treatment with pentylenetetrazole. Seizure susceptibility, an index of treatment safety, was studied by means of EEG, behaviour and hippocampus morphology. EEG spectral analysis was used as a bio-marker of the treatment.

KEY RESULTS

PTZ has a wide therapeutic window (0.03–3 mg·kg⁻¹) that is >10–1000-fold below its seizure threshold and chronic pentylenetetrazole treatment did not lower the seizure threshold. Short-term, low, chronic dose regimens of pentylenetetrazole elicited long-lasting (>1 week) normalization of cognitive function in young and aged mice. Pentylenetetrazole effectiveness was dependent on the time of treatment; cognitive performance improved after treatment during the light (inactive) phase, but not during the dark (active) phase. Chronic pentylenetetrazole treatment normalized EEG power spectra in TS mice.

CONCLUSIONS AND IMPLICATIONS

Low doses of pentylenetetrazole were safe, produced long-lasting cognitive improvements and have the potential of fulfilling an unmet therapeutic need in Down's syndrome.

Abbreviations

GABA_AR- α 5, GABA_A receptor containing α -5 subunits; LTP, long-term potentiation; MF, hippocampus granule cells mossy fibres; NOR, novel object recognition; TS, Ts65Dn

Introduction

Down's syndrome is caused by the triplication of all or part of chromosome 21 and is the most common genetic form of intellectual disability with a prevalence of approximately 1/750 live births (Morris and Alberman, 2009). The triplication of ~250 genes in Down's syndrome results in differential developmental and physiological effects (Nadel, 2003). Significant general health management improvements have substantially extended lifespan and quality of life of individuals with Down's syndrome (Yang *et al.*, 2002). Despite these advances, there are no drugs approved to address intellectual disabilities in Down's syndrome. Progress in understanding the molecular, cellular and functional impact of trisomy 21 (Hsa21) has come from the study of a growing number of mouse models that possess an extra copy of mouse genes syntenic to Hsa21 (Gardiner *et al.*, 2010). The best studied of these models is the Ts65Dn (TS) mouse that is trisomic for a segment of mouse chr16 that is orthologous to a region of HSA21 containing >150 genes (Davisson *et al.*, 1990). TS mice reproduce some key features of Down's syndrome including cognitive deficits (Gardiner *et al.*, 2010). Moreover, neural alterations occur in aged TS mice that resemble Alzheimer's disease, including abnormal processing of β -amyloid precursor protein and neuronal losses in basal forebrain and the locus coeruleus (Delcroix *et al.*, 2004; Salehi *et al.*, 2006; 2009).

Electrophysiological studies support the hypothesis that neuroplasticity dysfunction in TS mice is caused in part by enhanced inhibitory tone within neuronal circuits in brain regions such as the hippocampus. For example, hippocampal long-term potentiation (LTP) is impaired in TS mice (Siarey *et al.*, 1999; Kleschevnikov *et al.*, 2004; Costa and Grybko, 2005; Siarey *et al.*, 2006), and can be rescued by the acute application of GABA_A receptor antagonists, which also enhance LTP in normal animals (Bliss and Lomo, 1973; Arima-Yoshida *et al.*, 2011). At the behavioural level, studies showed memory enhancement in rats receiving non-convulsing doses of GABA_A receptor antagonists just before or immediately following training (Hunt and Krivanek, 1966; Elliott and Schneiderman, 1968; Krivanek and McGaugh, 1968; Hunt and Bauer, 1969; Krivanek, 1971; receptor nomenclature follows Alexander *et al.*, 2011).

These data suggest that low (non-epileptic) doses of GABA_A receptor antagonists might be an effective strategy to reducing elevated inhibitory tone and restore cognitive function in Down's syndrome. This concept was evaluated in a set of experiments demonstrating that a 2-week daily regimen of different GABA_A receptor antagonists -picrotoxin, bilobalide or pentylentetrazole – in low doses, normalized memory performance of these TS mice when they were tested either a week after or 2 months after the drug treatment (Fernandez *et al.*, 2007). In contrast, acute dosing of TS mice with low-dose picrotoxin (1 mg·kg⁻¹) 1 day before training did not improve performance of the animals in the novel object recognition (NOR) test. Similar results have been repeated by other laboratories (Rueda *et al.*, 2008) and even extended to models of Alzheimer's disease (Yoshiike *et al.*, 2008). Importantly, the pentylentetrazole regimen normalized hippocampal LTP in TS mice for up to 2 months after the treatment ended (Fernandez *et al.*, 2007).

These results indicate that short-term chronic treatments with GABA_A receptor antagonists can elicit long-lasting neuro-adaptive changes in neural circuit function and with subsequent behavioural benefits. They also raise questions of whether GABA_A receptor antagonists can be employed therapeutically in a safe and efficacious manner.

Of the several GABA_A receptor antagonists tested thus far, pentylentetrazole is of special interest. Pentylentetrazole was approved by the Food and Drug Administration (FDA) until 1982. Clinical studies assessed pentylentetrazole for pro-cognitive effects in various forms of neurological dysfunction in poorly characterized, senile geriatric populations (Gross and Finn, 1954; Aschenbach, 1956; Kapernick, 1957; Deitrick, 1967). These studies at least established the safety of long-term treatment with pentylentetrazole in man at doses significantly above those used in mice. The FDA removed pentylentetrazole from the list of approved drugs because of lack of evidence for efficacy in the treatment of any of several indications for which pentylentetrazole-containing drugs had been released (see Federal Register 47(86), 4 May 1982, pp 19208–19234).

The current study was designed to answer a number of questions about the therapeutic potential of pentylentetrazole to normalize cognitive function in young and old patients with Down's syndrome in a long-lasting manner. What is the minimum effective dose? What are the interactions of the drug with systems known to be involved in learning and memory such as the circadian system (Ruby *et al.*, 2008)? Is pentylentetrazole treatment safe in TS animals with regard to seizure activity? Is there a reliable EEG bio-marker associated with pentylentetrazole treatment?

Methods

Animals and genotyping

All animal care and experimental procedures were approved by the Stanford University Institutional Animal Care and Use Committee and complied with the NIH Guide for the Care and Use of Laboratory Animals. Particular efforts were made to minimize the number of animals used and the pain and distress they experienced. In addition, the ARRIVE guidelines were followed (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 240 mice were used for this study. Segmental trisomy 16 (TS) mice were obtained by mating female carriers of the 17¹⁶ chromosome (B6EiC3H – a/ATs65Dn) with C57BL/6J Ei × C3H/HeSnJ (C3H) F1 hybrid males (Reeves *et al.*, 1995) and produced either at Jackson West Laboratories, (Davis, CA, USA) or in our own colony. All mice used in our studies were male diploids (2N) controls and TS trisomy littermates on the B6/C3H background. Mice were maintained at 23 ± 2°C on a 12:12h light-dark schedule and had access to food and water *ad libitum*. Euploid and TS mice were genotyped using real-time quantitative PCR with *App*- and *Apob*-specific TaqMan probes (Applied Biosystems, Foster City, CA, USA). Mice carrying retinal degeneration (*Rd*) allele were excluded from experimental litters after genotyping (Blank *et al.*, 2011). In the following experiments, mice were randomly assigned to experimental groups.

Drug administration and scheduling

Pentylentetrazole (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in saline (NaCl) and diluted to the required concentration (injection volume was 0.1 mL per 30 g body weight). Drugs were given within 1 h at the middle of the light phase (6 h after light onset) except for the experiment in which treatment was given in the middle of the dark phase (6 h after dark onset). The chronic treatment protocol involved daily doses of pentylentetrazole or saline i.p. for 2 weeks followed by 8 days without treatment. After the non-treatment period (~1 week), mice were assessed for learning and memory using the NOR test, as described below. Independent groups of mice were studied for different dosages, age or circadian timing of treatment. In some experiments, pentylentetrazole was administered p.o. in chocolate milk as described previously (Fernandez *et al.*, 2007).

Behavioural testing

The behavioural test used in this study for assessing long-term memory was the NOR test (Dere *et al.*, 2007) carried out in arenas (50 × 50 × 50 cm) resting on a infrared emitting base. Behaviour was recorded by an infrared-sensitive camera placed 2.5 m above the arena. All behavioural testing was carried out within 2 h in the middle of the light phase (6 h after light onset). Data were stored and analysed using Videotrack software from ViewPoint Life Sciences, Inc. (Montreal, Canada) allowing the tracking of animals. On the day before NOR training, the mouse was habituated to the apparatus by freely exploring the open arena. NOR is based on the preference of mice for a novel object versus a familiar object when allowed to explore freely. For NOR training, two identical objects were placed into the arena and the animals were allowed to explore for 10 min. Testing occurred 24 h later in the same arena but one of the familiar objects used during training was replaced by a novel object (of similar dimensions and not generating spontaneous preference) and the animal was allowed to explore freely for 7 min. The objects and the arena were cleaned with 10% ethanol between trials. Exploration of the objects was defined by the time spent with the nose directed at the objects in a 2.5 cm zone around the objects. The discrimination index (DI) was calculated as the ratio of the time spent exploring the novel object over the total time spent exploring the two objects (additional descriptive data are given in the Supporting Information Table). The DI was calculated for each animal and averaged among the groups of mice by genotype/treatment/condition. The DI is non-significantly different from 50% in the training session, and is comparatively increased in the test session if novelty is detected (Ruby *et al.*, 2008).

EEG surgery and recordings

EEG recordings were used to assess the possible presence of epileptiform activity during the 2 weeks of treatment at 0.3 mg·kg⁻¹ and to assess possible subsequent changes in EEG characteristics during the behavioural testing time window, compared with pretreatment values. Surgical procedures were performed according to Colas *et al.* (2008) under deep anaesthesia (xylazine/ketamine i.p. at 10 and 3 mg·kg⁻¹ respectively; Ft. Dodge Animal Health, Ft. Dodge, IO and Akorn, Decatur, IL, USA) as assessed by the absence of response to hind paw pinch; post-operative analgesia was achieved using

carprofen (5 mg·kg⁻¹ s.c.; Pfizer, New York, NY, USA). Three-month-old TS and 2N mice were equipped with epidural EEG electrodes above the parietal cortex. Mice were allowed 2-weeks of recovery from surgery and habituation to experimental conditions before the experiments. Recordings were performed using an EMBLA™ amplifier and Somnologica-3™ (Ontario, Canada) software under standard conditions in individual cages. EEG signals were amplified, filtered (0.5–50 Hz), and analogue-to-digital converted at 200 Hz. The spectral analysis of the EEG was carried out using a 1024 Hz FFT on 200 Hz signals and 4 s windows. Spectrograms and power bands were computed for each animal then averaged within genotype and treatment groups.

Seizure threshold assessment

Seizure thresholds in 2N and TS mice were assessed in baseline or after treatment (pentylentetrazole 2 weeks daily i.p. 3 mg·kg⁻¹) by behaviour following the acute administration of 35 mg·kg⁻¹ pentylentetrazole (Marescaux *et al.*, 1984). The effect of a second higher acute dose of pentylentetrazole (45 mg·kg⁻¹; i.p.) was evaluated after an 8-day period. Seizure activity was quantified using a standard behavioural scoring over a period of 20 min following injections by two different observers who were unaware of the genotype or treatment: 1 = no response; 2 = staring, immobility; 3 = staring, rearing, nodding, and hind-limb pawing; 4 = forelimb clonus, rearing and falling; 5 = repeated forelimb clonus and falling; and 6 = status epilepticus and/or death. Scores 4–6 are equivalent to generalized clonic-tonic seizures with EEG discharges occurring in trains (Siemen *et al.*, 2011).

Histology of hippocampal mossy fibre MF sprouting following chronic pentylentetrazole treatment

Mouse hippocampal sections were prepared, processed and stained as described (Buckmaster and Lew, 2011), 10 days following a 3-week treatment of daily single doses of pentylentetrazole (3 mg·kg⁻¹, p.o.) in milk (Fernandez *et al.*, 2007). Mice were killed by an overdose of pentobarbital (200 mg·kg⁻¹ i.p.; Sigma-Aldrich, St. Louis, MO, USA) and perfused with 0.37% sodium sulfide followed by 4% paraformaldehyde. After post-fixation, hippocampi were isolated and placed in 30% sucrose. After equilibration, hippocampi were frozen and sectioned perpendicular to the septo-temporal axis at 30 µm. Starting at a random point near the septal pole, a one-in-six series of sections was mounted on slides, dried and developed for 45 mins in Timm stain (120 mL of 50% gum arabic, 20 mL 2 M citrate buffer, 60 mL 0.5 M hydroquinone and 1 mL of 19% silver nitrate). Timm staining of mice in the present study was compared with control and epileptic pilocarpine-treated mice from an earlier study (Buckmaster and Lew, 2011), which served as negative and positive controls respectively. MF sprouting was quantified by collecting bright field images on a Zeiss Axiovert microscope equipped with a 40× objective and Metamorph software (Molecular Devices, Sunnyvale, CA, USA). An investigator, unaware of the treatments, collected images of the granule cell and molecular layers of the dentate gyrus. MF collaterals were quantified by counting Timm positive axonal projections in a single field of view across six slices per animal then averaged within genotype and treatment conditions.

Statistical analysis

Data are presented as mean \pm SEM and were analysed using GraphPad Prism (San Jose, CA, USA). Data from the NOR test were analysed by comparing the mean DIs from testing sessions with those from training sessions, using a *t*-test for paired samples (Ruby *et al.*, 2008) for each genotype or treatment group. EEG power bands were analysed by a two-way ANOVA genotype \times treatment with Bonferroni *post hoc* tests. $P < 0.05$ was considered statistically significant.

Results

Chronic treatment with low doses of pentylenetetrazole leads to long-lasting improvements in learning in young TS mice

In an earlier study, we evaluated the pro-cognitive effect of p.o. dosing of pentylenetetrazole at 3 mg·kg⁻¹ using adult mice (6 months; Fernandez *et al.*, 2007). We therefore wanted to see if cognitive deficits were present in younger TS mice (2–3 months of age) and could be similarly treated with lower doses of pentylenetetrazole.

As before, mice received a chronic daily treatment protocol (2 weeks pentylenetetrazole or saline i.p., Figure 1A). Long-term memory in young (2–3 months old) mice was assessed 1 week later using the NOR test (Figure 1B). The 2N mice treated by this protocol show a strong preference for the novel object 24 h after the training session, as shown by a significantly greater DI (~60%) whether they had been treated with saline or pentylenetetrazole (Figure 1B-left). In contrast, TS mice treated with saline showed no ability to discriminate between the old and the novel object. TS treated with pentylenetetrazole (0.3 or 0.03 mg·kg⁻¹) displayed a clear ability to recognize the novel object (Figure 1B-right) as did 2N controls. However, when the dose was lowered to 0.01 mg·kg⁻¹, the TS mice showed no preference for the novel object.

Chronic pentylenetetrazole therapy improves cognitive function in aged animals

Knowing that important circuit remodelling occurs during aging in this model, we asked whether or not chronic pentylenetetrazole treatment would be effective in old TS mice. As in the previous protocol, we administered low doses of pentylenetetrazole (0.3 mg·kg⁻¹) once a day for 2 weeks to 12 to 15 months old 2N and TS mice (an advanced age for this genotype) and tested them ~1 week later (Figure 1C). Both genotypes showed impaired NOR behaviour when treated with saline, an expected result given the age of the mice. However, both the 2N and TS groups of mice treated with this low-dose regimen of pentylenetetrazole displayed a significantly increased DI in the testing versus training sessions. The pentylenetetrazole-treated 2N mice displayed a DI during the recognition test, similar to that in the 2N saline-treated group.

Efficacy of chronic pentylenetetrazole treatment is dependent on time of day

Performances on memory tasks show strong circadian patterns, and GABAergic activity also has daily rhythms (Ruby

et al., 2008). Therefore, we investigated whether the efficacy of the pentylenetetrazole treatment depended on the time of day. In accordance with the vast majority of the literature, drug dosing and behavioural testing in the previous experiments were done during the middle of the light period, the inactive phase in rodents. In 3 month old animals, we gave the chronic pentylenetetrazole treatment during the dark (active) phase at 0.3 mg·kg⁻¹ i.p. while maintaining NOR training and testing during the light phase. In contrast to treatment given during the light phase (Figure 1B-right), that given during the dark phase had no effect on DI patterns in TS mice (Figure 1D).

Taken together, these data indicated that short-term treatments with very low doses of pentylenetetrazole normalized long term memory in a sustained manner in young or aged TS mice and that this effect was dependent on the time of day when the treatment was given.

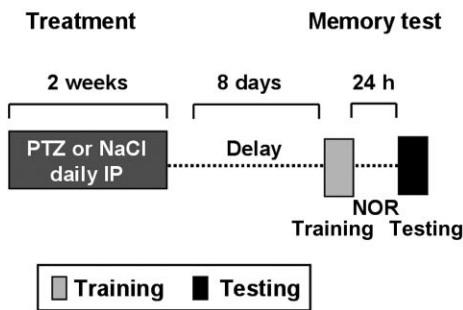
Absence of epileptogenicity signs related to pentylenetetrazole administrations

At high doses (>30 mg·kg⁻¹), pentylenetetrazole injections can elicit seizure activity and if repeated, can lower the threshold for seizure activity (kindling). We designed studies to confirm the safety and tolerability of pentylenetetrazole when used chronically at very low doses. Three experiments were performed in three independent groups of 3 month old animals.

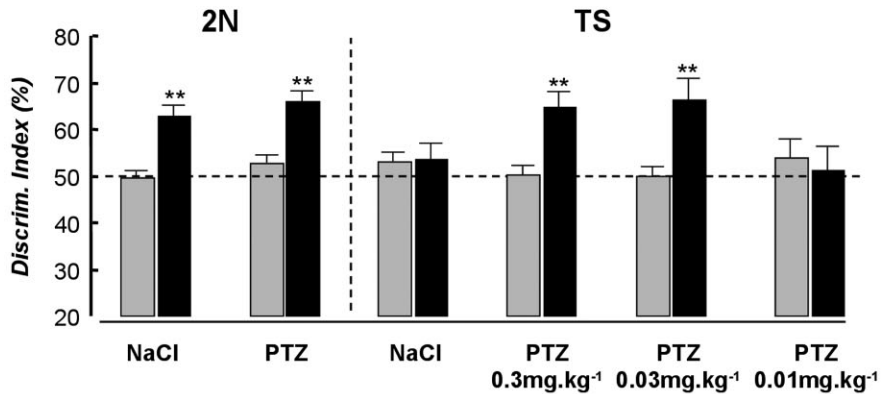
In the first group, EEG recordings were taken during the same protocol as in Figure 1A with pentylenetetrazole 0.3 mg·kg⁻¹ or saline (2N NaCl $n = 6$, TS NaCl $n = 5$, 2N pentylenetetrazole $n = 7$, TS pentylenetetrazole $n = 7$). Unlike some of our earlier studies in different models using a similar approach where we were able to detect discrete epileptiform features (Colas *et al.*, 2005; Siemen *et al.*, 2011), no evidence of seizures or spiking activity was detected here in either 2N or TS mice during the treatment. In particular, pentylenetetrazole did not elicit any acute 4–8 Hz bursts, characteristic of absence seizure during the hours following the drug administration. Typical TS EEG records are shown in Figure 2 and exhibited the expected waveforms corresponding to wakefulness (low amplitude traces) and sleep (high amplitude traces), typically seen during the light period in mice. Note that for both the beginning and end of the treatment period, no seizure activity was seen before and after pentylenetetrazole treatment.

In a second set of experiments (Figure 3), we examined whether TS mice had an increased susceptibility for chemically induced tonic-clonic seizures and/or whether a 2 week treatment with pentylenetetrazole (3 mg·kg⁻¹, the highest dose in the therapeutic window studied by Fernandez *et al.*, 2007) altered this sensitivity. We administered high single doses of pentylenetetrazole (35 and 45 mg·kg⁻¹ with 1 week in between doses) to 2N and TS animals that had or had not been treated chronically with low-dose pentylenetetrazole. Seizure severity was scored on a 1 to 6 scale with lower scores indicating a milder seizure and a score of 6 indicating a severe tonic-clonic seizure. Both challenge doses induced seizure activity but there were no significant differences between 2N and TS animals whether they had received the chronic low-dose pentylenetetrazole treatments or not.

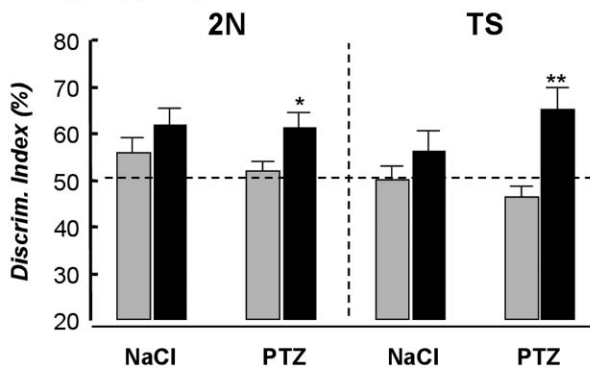
A Experimental design



B Treatment in young cohorts



C Aged group



D Dark period dosing

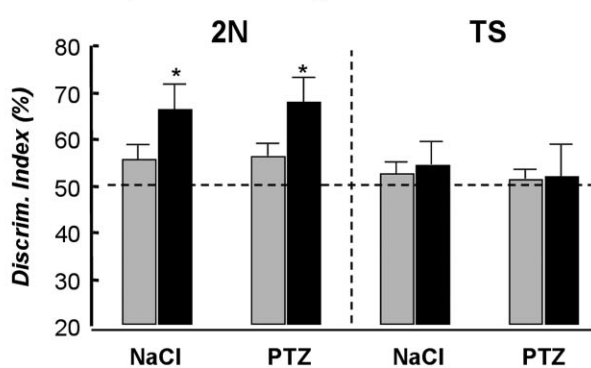


Figure 1

Long-lasting object memory normalization after low-dose pentylenetetrazole (PTZ) treatment in TS mice. (A) Pentylenetetrazole was given i.p. once daily for 2 weeks and mice were tested for long-term memory using the NOR test 1 week later. The corresponding DIs, as %, are shown as mean \pm SEM for training and testing sessions. A DI of ~50% indicates equal exploration of the two objects, and this characterizes the behaviour of the mice during training. If the mice remember the objects when they are tested 24 h later, they spend more time exploring the novel object, and the DI is significantly greater than the DI during training. (B) Three different doses of pentylenetetrazole (or NaCl as control) were given during the light period to separate groups of young (2–3 month old) mice. Data obtained in saline and pentylenetetrazole-treated 2N mice, and saline-treated TS mice were pooled from the three independent dose experiments: 2N NaCl $n = 23$, 2N pentylenetetrazole $n = 21$, TS NaCl $n = 14$, TS pentylenetetrazole 0.3 mg.kg⁻¹ $n = 7$, TS pentylenetetrazole 0.03 mg.kg⁻¹ $n = 7$, TS pentylenetetrazole 0.01 mg.kg⁻¹ $n = 6$. Pentylenetetrazole did not affect object memory in control diploid mice and the treatments with the two highest dosage normalized learning in TS mice. (C) Older animals (12–15 month old) were treated by the protocol defined in A using a pentylenetetrazole dosage of 0.3 mg.kg⁻¹ during the light period. 2N NaCl $n = 8$, 2N pentylenetetrazole $n = 13$, TS NaCl $n = 8$, TS pentylenetetrazole $n = 13$. Pentylenetetrazole improved learning in this older cohort. (D) Young mice were subjected to the protocol in A with a 0.3 mg.kg⁻¹ of pentylenetetrazole given during the dark period. 2N NaCl $n = 6$, 2N pentylenetetrazole $n = 6$, TS NaCl $n = 7$, TS pentylenetetrazole $n = 8$. Unlike the treatment given during the light period in B, treatment given during the dark period did not affect the long-term memory deficit characterizing TS mice. * $P < 0.05$, ** indicates $P < 0.01$; significantly different from training session; t -test for paired samples.

In a third set of experiments (Figure 4), we evaluated whether the chronic daily dosing of pentylenetetrazole at 3 mg.kg⁻¹ induced anatomical changes in brain circuitry indicative of kindling. A characteristic anatomical change accompanying kindling and/or recurrent tonic-clonic seizure is the inappropriate growth of granule cell MF axon collaterals in the molecular layer of the dentate gyrus (Sutula *et al.*, 1988). This was detected by using Timm stain to specifically label hippocampal granule cell MF axons, in 2N and TS animals 10 days following a 3-week treatment with pentylenetetrazole (3 mg.kg⁻¹, p.o.) (after Fernandez *et al.*, 2007). As shown in Figure 4A, in normal mice, seizures induced by a single dose of pilocarpine, which causes status epilepticus, result in a massive sprouting of granule cell MFs into the

molecular layer of the dentate gyrus. In contrast, the chronic treatment with pentylenetetrazole did not cause an increase in the number of MF axons within the granule cell and molecular layers of the dentate gyrus in either 2N or TS mice (samples are shown in Figure 4B and C respectively). The corresponding quantification of MF collaterals for each treatment or genotype group (Figure 4D) showed no difference between 2N and TS mice, with or without treatment.

Pentylenetetrazole treatment normalizes EEG power spectra

EEG recordings allow the quantification of brain rhythms that can serve as bio-markers. Our previous studies had identified EEG abnormalities such as increased spectral power in

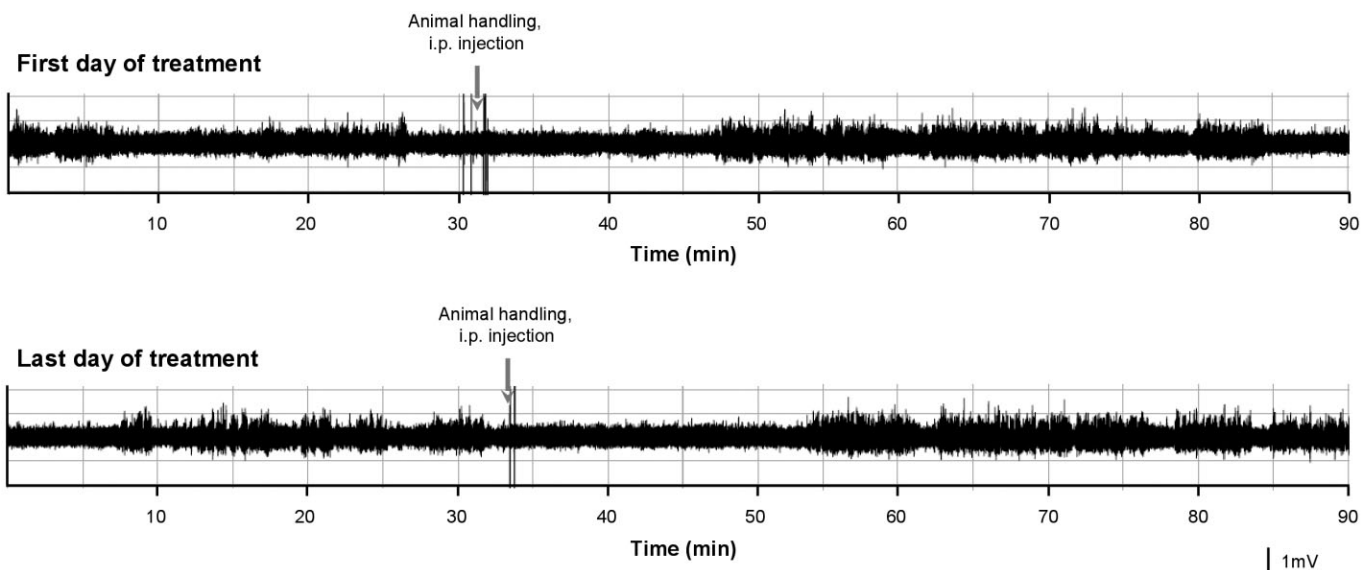


Figure 2

Low-dose chronic pentylenetetrazole (PTZ) treatment does not induce EEG signs of seizure activity. EEG samples from TS mice on the first (top trace) and last (bottom trace) days of a 2-week regimen of daily dosing with $0.3 \text{ mg}\cdot\text{kg}^{-1}$ pentylenetetrazole i.p. (arrow) are shown. Ninety consecutive minutes are displayed with 30 min before and 60 min after i.p. injection. Scale bar is 1 mV. Notice the normal power distribution and absence of spiking.

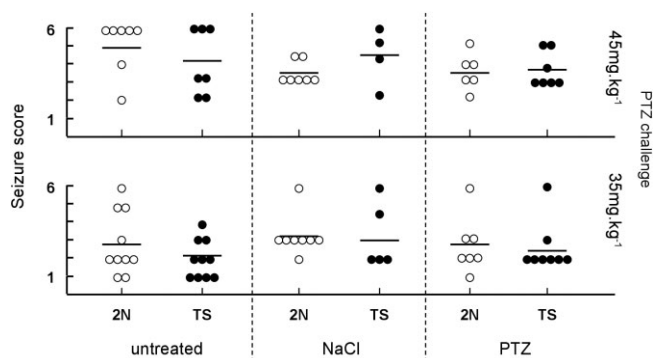


Figure 3

Behavioural assessment of seizure thresholds in young TS mice. Seizure thresholds were studied by mean of behavioural scoring after acute challenges with high doses of pentylenetetrazole. Three months old 2N/TS either naïve (untreated, left panel), or treated according to protocol in Figure 1A with NaCl (middle panel) or pentylenetetrazole $3 \text{ mg}\cdot\text{kg}^{-1}$ (right panel) were challenged with a single dose of $35 \text{ mg}\cdot\text{kg}^{-1}$ pentylenetetrazole (lower part) and 8 days later with a single dose of $45 \text{ mg}\cdot\text{kg}^{-1}$ pentylenetetrazole (upper part). Individual scores of seizure severity from 1 (moderate seizure) to 6 (severe tonic-clonic convulsions) are shown with a bar indicating the group average (initial group sizes were untreated: 2N $n = 10$, TS $n = 10$, NaCl: 2N $n = 8$, TS $n = 5$, pentylenetetrazole treated: 2N $n = 7$, TS $n = 8$). No differences are seen in seizure thresholds of the different groups of mice.

the θ band (6–10 Hz) in young TS mice (Colas *et al.*, 2008). We extended these previous studies to determine whether a behaviourally successful pentylenetetrazole treatment (2 weeks $0.3 \text{ mg}\cdot\text{kg}^{-1}$, i.p.) affected EEG dynamics. The mice

previously recorded (Figure 2) during this treatment and their controls (treated with saline) were recorded 1 week later in a follow-up study for 24 h corresponding to the time frame of cognitive improvements. The EEG recordings were used to identify epochs of wakefulness, and spectral analysis by fast Fourier transform yielded the corresponding power spectra (in $\text{mV}^2\cdot\text{Hz}^{-1}$) for each genotype or treatment group (Figure 5A). As previously seen, spectra from TS mice after saline treatment were characterized by increased EEG power, most noticeably in the θ band and encompassing higher frequencies. Further power band quantification (Figure 5B) and statistical analysis (two-way ANOVA with Bonferroni *post hoc* test) showed that TS mice given saline treatment were characterized by increased power in all frequency bands except δ (1–4 Hz), compared to saline-treated 2N mice. EEG power in pentylenetetrazole-treated TS mice was significantly reduced in a broadband manner. The θ (6–10 Hz), β (15–25 Hz) and γ (30–50 Hz) power bands were lowered in TS mice with pentylenetetrazole compared to TS with saline and were statistically not different from 2N mice with pentylenetetrazole treatment. The σ power band (12–15 Hz) was reduced in pentylenetetrazole-treated TS, compared with saline TS, mice and remained significantly higher than the corresponding value from pentylenetetrazole-treated 2N mice.

Discussion

Until recently, pharmacological treatments of cognitive deficits in individuals with Down's syndrome were considered unlikely due to the complexity of this disorder and the assumption that systems and circuits adversely affected during early development were beyond repair or modifica-

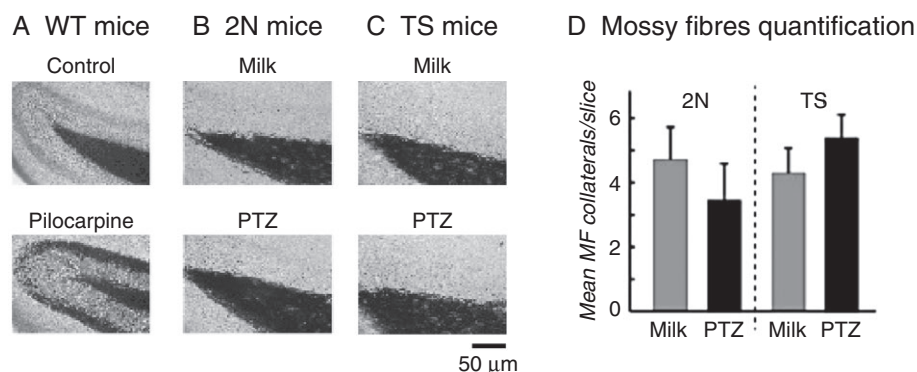


Figure 4

Pentylenetetrazole (PTZ) treatment does not induce histological signs of epilepsy. Timm staining was used to assess the presence of sprouting of granule cell MFs in the hippocampus, an index of history of seizure activity, 10 days after the following treatments. Young TS and 2N littermates were given 3 weeks of pentylenetetrazole (daily doses of $3 \text{ mg} \cdot \text{kg}^{-1}$) delivered p.o. in milk. Normal wild-type (WT; FVB/N) mice previously challenged with a single dose of pilocarpine were used as positive control. Panels A, B and C show photomicrographs of Timm staining of the dentate gyrus in WT, 2N or TS mice respectively (scale bar represents $50 \mu\text{m}$). The upper row shows examples of control sections (saline in WT or milk in 2N/TS mice). The lower row shows examples of sections from challenged mice (pilocarpine in WT mice as positive control or pentylenetetrazole in 2N/TS). The corresponding quantification is shown in panel D as mean \pm SEM MF collaterals per slice for $n = 6$ mice in each treatment or genotype group. Sprouting was marginal in all groups.

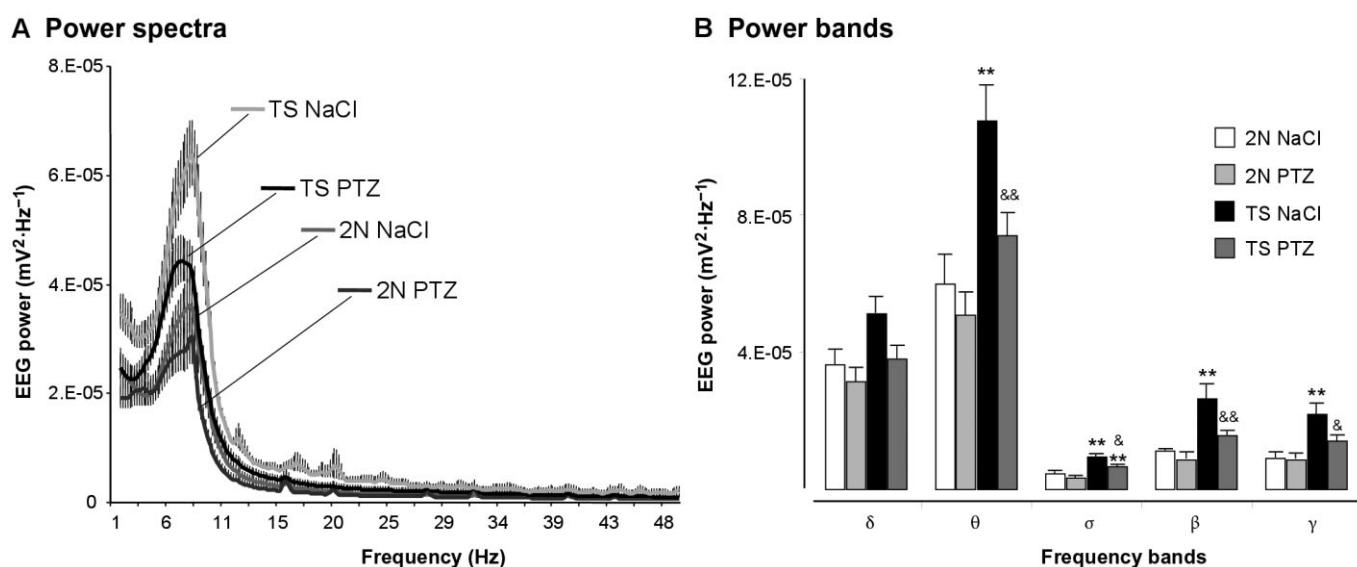


Figure 5

Pentylenetetrazole (PTZ) treatment normalizes the EEG power spectra of TS mice. EEGs were recorded for 24 h, 1 week after a $0.3 \text{ mg} \cdot \text{kg}^{-1}$ PTZ treatment and corresponding saline (SAL) controls in young TS and 2N mice. The spectral analysis of the EEG in awake mice was carried out using a 1024 Hz FFT for each animal then averaged within genotype and treatment groups (2N NaCl $n = 6$, TS NaCl $n = 4$, 2N pentylenetetrazole $n = 7$, TS pentylenetetrazole $n = 5$). The resulting power spectra are shown in panel A as mean power ($\text{mV}^2 \cdot \text{Hz}^{-1}$) \pm SEM for each frequency bin of 0.2 Hz increment from 1 to 50 Hz. Power bands were calculated from individual spectrograms as δ 1–4 Hz, θ 6–10 Hz, σ 12–15 Hz, β 15–25 Hz and γ 30–50 Hz then averaged and shown as mean \pm SEM in panel B for each genotype and treatment group. We wanted to test whether the treatment affected one genotype and whether the genotypes were different after pentylenetetrazole. The broadband EEG power increase characterizing TS mice was reduced by pentylenetetrazole treatment. * $P < 0.05$, ** $P < 0.01$; significantly different for genotype within a treatment group: & $P < 0.05$, && $P < 0.01$; significantly different for treatment within a genotype; two-way ANOVA genotype \times treatment with Bonferroni *post hoc* tests.

tion in children. Studies in mouse models of Down's syndrome, however, suggest that many affected brain circuits could be responsive to pharmacotherapies designed to restore equilibrium through adaptive changes affecting attention or

cognition (Costa and Grybko, 2005; Fernandez *et al.*, 2007; Rueda *et al.*, 2008; Salehi *et al.*, 2009; Faizi *et al.*, 2011). Despite the caveats associated with disease modelling, those studies could lead to key clinical developments. In the

current study, we explored the therapeutic potential of pentylenetetrazole, a GABA_A receptor antagonist, for mildly modulating brain inhibition.

Analyses of mouse models of Down's syndrome have shown that a number of neurotransmitter systems are altered by the triplication of subsets of genes syntenic to Hsa21 genes. In young animals, the most prominent abnormalities are associated with increases in GABAergic signalling and a concomitant decrease in glutamatergic transmission, resulting in an altered excitatory to inhibitory balance and impairing LTP in the hippocampus (Siarey *et al.*, 1999; Belichenko *et al.*, 2004; Kleschevnikov *et al.*, 2004; Costa and Grybko, 2005; Siarey *et al.*, 2006; Belichenko *et al.*, 2009). Translating this concept in a therapeutic perspective, we found that low doses of a variety of GABA_A receptor antagonists given chronically, could restore LTP and learning by mildly reducing inhibitory drive (Fernandez *et al.*, 2007). Importantly, improved learning was maintained after the pharmacotherapy ended, suggesting that low doses of GABA_A receptor antagonists given repeatedly could mediate stable changes in neuronal circuit functions.

Of the antagonists examined previously, pentylenetetrazole is most promising for future assessment in man, because of its long history of safe clinical use. For instance, daily oral doses of 100–400 mg were used in elderly patients for dementia symptoms (Chesrow *et al.*, 1951). Thus, though pentylenetetrazole is no longer approved by the FDA, it offers the possibility of being reassessed for specifically addressing learning disabilities in individuals with Down's syndrome. To further explore this possibility, we sought first to define the potential therapeutic window for a pentylenetetrazole low-dose chronic regimen. Including our earlier study, we found that doses from 0.03 to 3 mg·kg⁻¹ were able to restore long-term object memory in TS mice. While the highest dose induced improvement lasting a month or longer (Fernandez *et al.*, 2007), the benefits with lower doses lasted at least a week. These data suggest that chronic periodic regimens of pentylenetetrazole at doses 10–1000-fold below the pro-convulsive threshold in mice could be an efficacious pro-cognitive strategy.

Age-related changes in neuronal circuits occur rapidly in Ts65D mice and our previous studies used adult (5–6 month old) mice or, as in the present study, in young (2–3 month old) animals. We extended those findings showing that the low-dose (0.3 mg·kg⁻¹) chronic regimen restored long-term object memory in aged (12–15 month old) TS mice. Aged mice (>12 months for TS) are of particular interest as neurodegenerative phenotypes, resembling Alzheimer's disease, appear from 6 months of age in TS mice (Salehi *et al.*, 2006; 2009). Of note, studies on mouse models of Alzheimer's disease have similarly found that a three-week, low dose of the GABA_A receptor antagonist, picrotoxin, normalized cognition (Yoshiike *et al.*, 2008). This is of particular interest to the Down's syndrome community as the occurrence of Alzheimer's disease pathology in older individuals with Down's syndrome is an important feature (Rissman and Mobley, 2011).

As with our earlier study, the long-lasting effect of the chronic pentylenetetrazole therapy was striking and different in nature from acute approaches, which elicit immediate but not long-lasting benefits. Given that we are using a GABA_A

receptor antagonist, it seems reasonable to conclude that these drugs improve memory by stably reducing inhibitory tone within the circuits involved in memory consolidation. However, the chronic treatment regimen does not allow us to dissect other possible mechanisms. Conceptually, if excessive inhibition blocks either the acquisition and/or storage of information, then the acute administration of these drugs during training should improve learning. In earlier work, we did not find a pro-cognitive effect of an acute single dose of pentylenetetrazole given 1 day before training (Fernandez *et al.*, 2007). However, many other studies, for instance (Hunt and Krivanek, 1966; Elliott and Schneiderman, 1968; Krivanek and McGaugh, 1968; Hunt and Bauer, 1969; Krivanek, 1971) have shown that enhancing effects of GABA_A receptor antagonists (including pentylenetetrazole) on long-term memory in normal mice or rats requires administration of the drug shortly before or after training (± 15 min). When we attempted to replicate this protocol (see Supporting Information Figure S1), we found that acute doses of pentylenetetrazole (3 mg·kg⁻¹) given 10 min before training enhanced long-term memory in the young TS mice, but that this effect was not long lasting. We believe that in this paradigm, pentylenetetrazole may have directly enhanced LTP as predicted from numerous studies showing the role of inhibition on neuronal plasticity (Kleschevnikov *et al.*, 2004; Arima-Yoshida *et al.*, 2011). In contrast, a single acute dose of 0.3 mg·kg⁻¹ failed to improve long-term memory (Supporting Information Figure S1) showing that the sensitivity of the TS mice to the chronic treatment with pentylenetetrazole is also greater than that to the acute treatment. Nevertheless, these results support our general hypothesis that, in TS mice, enhanced inhibitory tone is responsible for the learning disability and slight reduction of that tone with an acute dose of a GABA_A receptor antagonist during a cognitive task enables learning. These findings are paralleled by recent studies using specific antagonists for GABA_A receptors containing α -5 subunits (GABA_AR- α 5). Indeed, an acute treatment with a GABA_AR- α 5 antagonist prior to training improved long-term memory in 3 month old TS mice but no long-lasting effect was reported (Braudeau *et al.*, 2011). Pentylenetetrazole is a non-specific GABA_A receptor antagonist and would be expected to inhibit all GABA_A receptor subtypes, including those containing α -5 subunits. At present, it is unclear how or if GABA_AR- α 5 may be affected by our low-dose regimen and whether other mechanisms may be important in triggering the long-lasting effects of pentylenetetrazole pharmacotherapy. Our pharmacokinetic data (see Supporting Information Figure S2) show that the drug half-life at the proposed therapeutic window is less than 5 h. Thus, daily dosing is unlikely to cause accumulation of the drug over time. The drug would therefore not be present when the delayed behavioural testing was done. Thus, we postulate that the chronic pentylenetetrazole therapy has in some way led to a long-lasting circuit adaptation or reduced the GABA sensitivity of key circuits involved in learning and memory.

A possible clue to the identity of a GABAergic system involved in mediating the sustained effects of the chronic pentylenetetrazole treatment comes from our results relating efficacy to the time of day of the treatment. The dosing protocol that enabled memory in the TS mouse when administered during the light phase had absolutely no effect when

delivered during the dark (active) phase. This finding might be of importance in adjusting the proposed treatment according to circadian timing in clinical trials. It also indicates a possible involvement of circadian-modulated processes and/or sleep in the long-lasting improvement in the function of memory circuits (Holloway and Wansley, 1973a,b; Ruby *et al.*, 2008; Diekelmann and Born, 2010). Sleep architecture was not changed by the chronic pentylentetrazole treatment (Supporting Information Table S2) in our experiments, but pentylentetrazole may affect sleep-related neuroplasticity processes (Wang *et al.*, 2012). The master circadian clock in mammals is the suprachiasmatic nucleus (SCN), which is predominantly a GABAergic nucleus (Moore and Speh, 1993). Moreover, the neural activity of the SCN is high during the light phase and low during the dark phase. In a study of circadian rhythms of TS mice, we found that these animals had greater power in their circadian rhythms than did the wild-type controls (Ruby *et al.*, 2010). It is thus possible that the SCN of TS mice, compared with that of 2N mice, has higher levels of activity during the light phase resulting in higher levels of GABA release from efferent pathways that might influence learning and memory, making this phase more sensitive to pentylentetrazole. This possibility gains support from experiments on Siberian hamsters that can be made arrhythmic by a simple, one-time manipulation of their light/dark cycle that leaves the SCN intact but continuously, rather than cyclically, active (Ruby *et al.*, 2008). These animals exhibit deficits in the NOR test but learning is restored by the same pentylentetrazole protocol as described in this paper, without resolving their circadian arrhythmicity.

Pentylentetrazole at high doses ($>30 \text{ mg}\cdot\text{kg}^{-1}$ in mice) is convulsive but such doses are significantly higher (10–1000-fold) than those required here for successful pharmacotherapy ($0.03\text{--}3 \text{ mg}\cdot\text{kg}^{-1}$). To confirm safety, we conducted several studies to assess whether this drug induced epileptogenic activity or affected seizure thresholds. In our EEG recordings of TS and 2N mice treated chronically with pentylentetrazole ($0.3 \text{ mg}\cdot\text{kg}^{-1}$), no indications of epileptiform activity were observed. A corollary of seizure activity is MF sprouting in the dentate gyrus, as seen in the pilocarpine model. No significant sprouting of MF was observed in TS or 2N mice receiving the chronic pentylentetrazole protocol at the highest chronic concentrations used ($3 \text{ mg}\cdot\text{kg}^{-1}$). Finally, we used convulsive doses of pentylentetrazole to evaluate whether TS mice were more susceptible to chemically induced seizure than 2N mice and whether chronic pentylentetrazole treatment at $0.3 \text{ mg}\cdot\text{kg}^{-1}$ would increase their sensitivity. No differences in seizure responses of 2N and TS mice were observed. These results fit with the long history of safe, chronic use of pentylentetrazole in humans (Gross and Finn, 1954; Aschenbach, 1956; Kapernick, 1957; Deitrick, 1967). Clinical studies conducted in the 1950s and 1960s used pentylentetrazole to treat patients with senile dementia. Although efficacy in treating the symptoms was inconsistent, no issues of safety or tolerability were raised even though the patient population had a great diversity of comorbidities and were taking pentylentetrazole at doses of hundreds of $\text{mg}\cdot\text{day}^{-1}$ for months to years. For example, one study enrolled 687 patients who took $600 \text{ mg}\cdot\text{day}^{-1}$ for 1 to 10 years (Kapernick, 1957). No adverse effects were reported. These doses are significantly above what we predict would be thera-

peutic in human patients with Down's syndrome, further supporting the feasibility of using pentylentetrazole safely.

We were interested in providing possible biomarkers for future translational studies, and EEG techniques can be used routinely in preclinical studies. The present data, in line with an earlier study (Colas *et al.*, 2008), revealed that the EEG profile in TS mice was clearly abnormal showing increased power across a broad range of frequencies. Interestingly, this specific feature has been consistently described in patients with Down's syndrome (Politoff *et al.*, 1996; Babiloni *et al.*, 2010; Velikova *et al.*, 2011). A mouse model of Alzheimer's disease characterized by an altered cortical excitation–inhibition balance shows a similar broadband EEG power increase (Gurevicius *et al.*, 2012). We thus believe this feature to be an important marker of the condition. Interestingly the pentylentetrazole treatment had a broadband effect on EEG power, which could therefore be used as a bio-marker for clinical studies. The mechanistic insights given by an integrated parameter such as the EEG could be of importance too. Indeed, the physical properties of the EEG result mostly from the underlying cortical somato-dendritic arrangements (Buzsaki *et al.*, 2012) that can be altered by moderate genetic imbalances such as those seen in intellectual disabilities including Down's syndrome (Verpelli and Sala, 2011; Levenaga and Willemsen, 2012). In particular, modest changes of neuronal units can result in significant alterations at a larger scale such as the EEG. In turn, EEG normalization resulting from the treatment observed in our study could be linked to subtle changes due to that treatment at the synptomorphological level and be a quantitative sign of circuit adaptation.

We conclude that pentylentetrazole is an excellent drug candidate for the treatment of learning impairments in the TS mouse model of Down's syndrome. We acknowledge that the TS mouse is not a perfect model of trisomy 21, but it has been very useful in investigations of possible causes and remedies for the cognitive impairment associated with Down's syndrome. Results from this mouse model encourage further preclinical research with more recent and more complete mouse models of Down's syndrome such as the Dup(1)16Yey mouse (Yu *et al.*, 2010). Our results show that very low doses of pentylentetrazole safely elicit pro-cognitive effects over a long period of time in a circadian sensitive way. Our data further suggest that circuits involved in memory processing can be rearranged, in a sustained manner, by repeated exposure to GABA_A receptor antagonists.

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Conflict of interest

C. C. Garner and D. Z. Wetmore are co-founders of Balance Therapeutics, which has taken a license from Stanford University to develop pentylentetrazole as a pharmacotherapy for cognitive dysfunction of Down's syndrome.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Short term effects of acute pentylentetrazole treatment on NOR in Ts65Dn mice.

Figure S2 Pharmacokinetics. Blood levels of pentylentetrazole in rats following iv, ip, or oral administration.

Table S1 Locomotor and exploratory behaviour in NOR training sessions.

Table S2 Sleep architecture (min/12 h) after pentylentetrazole treatment.